



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b>  <b>C12N 5/02, 5/06</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/02999</b>  <b>(43) International Publication Date:</b> 20 January 2000 (20.01.00)
<b>(21) International Application Number:</b> PCT/US99/15464  <b>(22) International Filing Date:</b> 9 July 1999 (09.07.99)  <b>(30) Priority Data:</b> 09/113,437      10 July 1998 (10.07.98)      US  <b>(71) Applicant (for all designated States except US):</b> ENCELLE, INC. [US/US]; 2408 S. Charles Boulevard, Greenville, NC 27858 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> USALA, Anton-Lewis [US/US]; 237 Buckingham Drive, Winterville, NC 27890 (US). KLANN, Richard, Chris [US/US]; 239 East Main Street, Washington, NC 27889 (US).  <b>(74) Agents:</b> HUMPHREY, Christopher, M. et al.; Alston & Bird LLP, P.O. Drawer 34009, Charlotte, NC 28234-4009 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> MEDIUM AND MATRIX FOR LONG-TERM PROLIFERATION OF CELLS  <b>(57) Abstract</b>  <p>A cell culture medium and hydrogel matrix for long term storage and proliferation of cells is provided. The cell culture medium and hydrogel matrix may include an effective amount of polar amino acids, the polar amino acids selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid. One embodiment of the cell culture medium comprises about 5 to about 150 mM of polar amino acids. The hydrogel matrix comprises about 3 to about 150 mM of polar amino acids. L-arginine and L-glutamic acid are preferably supplemented in the cell culture medium. L-arginine, L-lysine, and L-glutamic acid are preferably supplemented in the hydrogel matrix. A method of maintaining viability and functioning of a transplant is also provided. The method of maintaining viability of a transplant includes encapsulating the cells in a hydrogel matrix and injecting the encapsulated cells into the host organism. The matrix of the present invention may also be used to promote vascularization in a transplant site prior to injection of cells.</p>		

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## MEDIUM AND MATRIX FOR LONG-TERM PROLIFERATION OF CELLS

### FIELD OF INVENTION

The present invention relates to a cell culture medium and matrix composition for preserving cell viability as well as gene expression and specialized tissue function. The present invention also relates to a matrix  
5 capable of sustaining cell viability after injection of hormone secreting cellular moieties into living tissue.

### BACKGROUND OF THE INVENTION

New methods for treating insulin-dependant diabetes mellitus are  
10 presently being sought. At the present time, diabetes patients test their blood sugar levels and inject insulin when necessary. Although it is possible to transplant a pancreas from one human to another, the survival rate for this procedure is only 40% at one year following surgery. Researchers have used isolated pancreatic islets in transplantation approaches in attempts to find a  
15 viable long term treatment of diabetes.

The islets of Langerhans are clusters of differentiated cells sharing a common precursor. Found in the pancreas of mammals, islets taken together can be considered as a single endocrine organ. The islets occupy about 7% of the human pancreas which also contains the exocrine acinar tissue. The  
20 composition of cells in the islets differs depending on the location of the islet in the pancreas. Central to each islet is a core of insulin secreting beta cells. Surrounding the beta cells are somatostatin secreting delta cells, glucagon secreting alpha cells and pancreatic polypeptide containing f cells. Alpha cells tend to be concentrated in the tail and the body of the pancreas whereas, the f  
25 cells are concentrated in the head. This distribution corresponds to the embryonic origin of alpha and f cells from dorsal and ventral primordium of the pancreas.

Pancreatic beta cells are the only cells in which the insulin gene is

expressed and, therefore, are the sole source of metabolic insulin in vertebrates. Insulin is necessary in maintaining glucose homeostasis and plays a role in the normal processing of proteins and fats. Insulin release can be inhibited by low levels of somatostatin and stimulated by glucagon. Without  
5 sufficient insulin to metabolize glucose, hyperglycemia occurs. Insulin-dependant diabetes mellitus is a direct result of nonfunctional islets, specifically beta cells.

Among the major obstacles in islet transplantation research is an inability to induce proliferation and to keep islets viable over time.  
10 Researchers have encountered many obstacles in attempting to cure diabetes resulting from the loss of islet function. For transplantation, it is necessary to preserve islet viability as well as gene expression and secretory function.

Pancreatic islets do not grow readily in primary cultures. However, these endocrine cells have been grown with difficulty as monolayers. This  
15 difficulty of long-term culture has not only hindered the laboratory research for such islets, but it has also hindered attempts to carry out physiological and even clinical studies with such islets. Therefore, there is needed a medium for the long-term proliferation of islets. A medium for the long-term survival of cells is additionally needed for other cell types.

20 Additionally, current methods of transplantation must suppress immune response by the host organism that may lead to rejection of the transplanted cells and loss of islet function. Thus, there is also a need in the art for a simple, non-invasive method of introducing hormone secreting cellular moieties, such as insulin secreting pancreatic islets, into a hormone deficient  
25 organism without requiring general immunosuppressive agents.

### SUMMARY OF THE INVENTION

A cell culture medium to promote the proliferation and long-term survival of cells is provided. In one embodiment, the cell culture medium  
30 includes elevated levels of polar amino acids. The addition of polar amino

acids to the medium enhances cell proliferation and maintains cell viability for sustained periods of time. In another embodiment, the cell culture medium includes at least one nitric oxide inhibitor.

5 Additionally, a hydrogel matrix for the long-term proliferation of cells is provided. The matrix includes elevated levels of polar amino acids. The matrix of the present invention may be used as a carrier for direct injection of cells into a host organism without significant loss of cell viability or function. Additionally, the matrix acts to shield the cells from the immune system of the host organism.

10 Also provided are transplants capable of long-term functioning in a host. In particular, insulin secreting transplants comprising islet cells and acinar cells are provided. The transplants of the present invention include the matrix and allows coexistence of islet and acinar cells with improved insulin pulsatility.

15 The cell culture medium includes an effective amount of polar amino acids. Preferred polar amino acids are selected from, but not limited to, the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid. The effective amount of polar amino acids is preferably about 5 to about 150 mM and most preferably about 10 to about 64 mM. In one embodiment,  
20 the polar amino acids comprise about 2 to about 60 mM of arginine and about 2 to about 60 mM of L-glutamic acid. The cells cultured in the medium may be selected from a group consisting of lung cells, liver cells, kidney cells, thymus cells, thyroid cells, heart cells, brain cells, pancreatic islet cells, pancreatic acinar cells, and mixtures thereof.

25 A hydrogel matrix for long term storage and proliferation of cellular tissue is also provided, the matrix comprising about 0.01 to about 40 mM of gelatin and an effective amount of polar amino acids. The effective amount of polar amino acids is preferably from about 3 to about 150 mM and most preferably about 10 to about 65 mM. In one embodiment, the polar amino  
30 acids are selected from the group consisting of arginine, glutamic acid, lysine

or mixtures thereof. Preferably, the hydrogel matrix includes about 2 to about 60 mM of L-glutamic acid, about 1.5 to about 10 mM of L-lysine and about 1 to about 40 mM of arginine.

5 A method of maintaining cell viability and functioning during storage is provided wherein the cells are imbedded in the hydrogel matrix of the present invention. The matrix protects cells during storage, including frozen storage.

10 A transplant for implanting in a host organism is also provided. The transplant comprises cells having outer surfaces encapsulated by a matrix comprising an effective amount of polar amino acids. The effective amount of polar amino acids may be about 3 to about 150 mM. These polar amino acids serve to enhance bonding of other polar moieties and further obscure immune recognition proteins in a host subject. Thus, cells embedded in this enhanced hydrogel matrix substantially escape host immune destruction.

15 A method for increasing insulin production in a transplant is also provided. Insulin production may be increased in a transplant by providing a mixture of acinar cells and islet cells and encapsulating that mixture in a matrix comprising an effective amount of polar amino acids to form a transplant. The transplant is then injected into a host organism. Preferably  
20 the mixture of acinar cells and islet cells comprises at least about 30% by volume acinar cells and most preferably about 60% by volume acinar cells.

A method of metabolically refeeding stored cells is also part of the present invention. Stored cells may be refed by providing a container of stored cells at room temperature and adding cell culture medium of the present  
25 invention to the container. The container of stored cells is then incubated for a period of time. Advantageously, the cell culture medium is added in an amount equal to about 10 to about 40  $\mu$ l/ml of stored cells.

A method of protecting cells during isolation of the cells after enzymatic digestion of cell tissue is also included in the present invention.  
30 The method includes the steps of collecting digestate from a digestion process

and adding cell culture medium of the present invention to the digestate to protect cells during isolation.

#### BRIEF DESCRIPTION OF THE DRAWINGS

5 Having thus described the invention in general terms, reference will now be made to the accompanying drawings, wherein:

Figure 1 is a table listing the amount of porcine c-peptide produced by three separately designated animals;

10 Figure 2 is a set of three graphs indicating the relationship between insulin production and islet purity;

Figure 3 is a table indicating insulin collection as a function of islet number, purity and age; and

Figure 4 is a bar chart showing the relationship between the blood glucose levels of two dogs.

15

#### DETAILED DESCRIPTION OF THE INVENTION

The invention comprises compositions and methods useful for making and using transplants. The invention also comprises compositions and methods of maintaining cell viability and function over long periods of time.

20 Specifically, the invention provides a cell culture medium composition and matrix composition that facilitates long-term storage and transplantation of cells.

By transplant is intended cells, tissues, or other living or non-living devices for transplantation into a mammal. Transplants of the invention  
25 include allografts, artificial organs, cellular transplantation and other applications for hormone producing or tissue producing implantation into deficient individuals who suffer from conditions such as diabetes, thyroid deficiency, growth hormone deficiency, congenital adrenal hyperplasia, Parkinson's disease, and the like. Likewise, the matrix is useful for  
30 transplants involving therapeutic conditions benefiting from implantable

delivery systems for biologically active and gene therapy products for the treatment of central nervous system diseases and other chronic disorders.

More specifically, the matrix as described will find application in the various transplantation therapies, including without limitation cells secreting human  
5 nerve growth factors for preventing the loss of degenerating cholinergic neurons, satellite cells for myocardial regeneration, striatal brain tissue for Huntington's disease, liver cells, bone marrow cells, dopamine-rich brain tissue and cells for Parkinson's disease, cholinergic-rich nervous system for Alzheimer's disease, adrenal chromaffin cells for delivering analgesics to the  
10 central nervous system, cultured epithelium for skin grafts, and cells releasing ciliary neurotropic factor for amyotrophic lateral sclerosis, and the like.

#### Cell Culture Medium

In order to cultivate animal cells *in vitro*, conditions such as those  
15 found *in vivo* must be reproduced as closely as possible. These conditions are affected by numerous factors, including: temperature, pH, osmotic pressure, cell growth matrix, essential metabolites, supplemental metabolites, hormones, and specific factors for cell metabolism such as transport factors, antibiotics, etc.

20 A medium for the long-term survival and proliferation of cells is provided. In general, the terms "medium" and "media" in connection with the present invention are solutions containing growth factors and nutrients which are used to support the growth and development of cells, particularly islet cells. By "long-term" is meant continuous growth and development of the  
25 cells being cultured, for a time period of at least about 12 to about 20 weeks, preferably greater than 20 weeks, and more preferably greater than 40 weeks.

The medium of the invention is useful for the growth and proliferation of a variety of cells. Such cells may be derived from a variety of tissues such as lung, liver, kidney, thymus, thyroid, heart, brain, pancreas, and the like, as  
30 well as various cultured cell populations.



In one embodiment, a number of amino acids are included within the medium. Of particular interest are polar amino acids, particularly arginine and glutamic acid. By amino acid is intended all naturally occurring alpha amino acids in both their D and L stereoisomeric forms, and their analogues and derivatives. An analog is defined as a substitution of an atom in the amino acid with a different atom that usually has similar properties. A derivative is defined as an amino acid that has another molecule or atom attached to it. Derivatives would include, for example, acetylation of an amino group, amination of a carboxyl group, or oxidation of the sulfur residues of two cysteine materials to form cystine.

The addition of supplemental amounts of polar amino acids is an important feature of one embodiment of the cell culture medium of the present invention. While the invention is not bound by any particular mechanism, it is believed that the polar amino acids strengthen cellular membranes by binding to polar groups found on the cellular membrane surface. This increases the integrity of the cellular membrane and protects the cell from trauma in the culture medium environment. Additionally, the polar amino acids may bond to immune recognition sites on the cell surface which suppresses adverse immune responses.

The concentration of polar amino acids may be raised until an effective amount of polar amino acids are present in the culture medium. The preferred polar amino acids are selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid, although other chemicals containing polar amine and carbonyl groups may be used. An effective amount is the amount necessary to strengthen cellular membranes and bond to immune recognition sites on the cell surface. In one embodiment, the concentration of polar amino acids is raised to a final concentration of between about 5 to about 150 mM, preferably about 10 to about 65 mM, and more preferably about 15 to about 40 mM.

Advantageously, supplemental amounts of L-arginine and L-glutamic

acid are added to the culture medium of the present invention. Preferably, the final concentration of L-arginine is about 2 to about 60 mM, preferably about 5 to about 30 mM, most preferably about 5 to about 15 mM. The final concentration of L-glutamic acid is about 2 to about 60 mM, preferably about 5 to about 30 mM, most preferably about 10 to about 20 mM. In one embodiment, the final concentration of arginine is about 10 mM and the final concentration of L-glutamic acid is about 15 mM.

The cell culture medium may also be used to protect cells during an isolation process following digestion of cellular tissue. By adding the cell culture medium to the digestate, the cells are protected from digestion, the mechanical trauma caused by the isolation process, and later, after mixing with serum, attack by high affinity antibodies. The result is less cell fragmentation during isolation.

In addition to supplemental amounts of polar amino acids, the culture medium of the present invention also comprises a standard culture medium supplemented with a buffering agent, salt solution and other additives. The preferred standard culture medium is Medium 199 1x liquid. However, other standard culture media known in the art would be suitable for use with the present invention. Standard culture media which may be employed in accordance with the present invention are standard culture media for growing cells that typically provide an energy source, such as glucose, substantially all essential and nonessential amino acids and vitamins and/or other cell growth supporting organic compounds required at low concentrations. When combined with a buffering agent and a salt solution, the standard culture medium provides many of the nutrients required for normal metabolic functioning of cultured cells.

The preferred salt solution is Earle's Balanced Salts. The salt solution helps to maintain pH and osmotic pressure and also provides a source of energy. The preferred buffering agent is Hepes. Other salt solutions and buffering agents known in the art may be used without departing from the

present invention.

Table 1 below lists the particularly preferred components along with preferred approximate concentrations for each component of a solution containing a standard culture medium, buffering agent and salt solution. The concentrations are based on use of Medium 199 liquid, Earle's Balanced Salts and Hepes.

Table 1

Component	Preferred Concentration (mM)
<b>Inorganic Salts</b>	
Calcium Chloride	1.26000
Potassium chloride	5.34000
Potassium phosphate	0.43900
Magnesium sulfate * 7H <sub>2</sub> O	0.83074
Sodium chloride	90.48920
sodium bicarbonate	4.14050
sodium phosphate	0.38500
ferric nitrate *9H <sub>2</sub> O	0.00170
sodium acetate	0.62000
<b>Amino Acids</b>	
Cystine	0.08300
L-alanine	0.11670
L-arginine HCl	0.39861
L-aspartic acid	0.23000
L-cysteine HCl *2H <sub>2</sub> O	0.00057
L-glutamic acid	0.51000
L-glutamine	0.68400
glycine	0.53674
L-histidine HCl H <sub>2</sub> O	0.12935

Component	Preferred Concentration (mM)
L-hydroxyproline	0.07600
L-isoleucine	0.33957
L-leucine	0.58299
L-lysine HCl	0.52630
L-methionine	0.07424
L-phenylalanine	0.17424
L-proline	0.34497
L-serine	0.28878
L-threonine	0.08936
L-tryptophan	0.05860
L-tyrosine 2Na*2H <sub>2</sub> O	0.44372
L-valine	0.09974
<b>Vitamins</b>	
ascorbic acid (vitamin C)	0.00233
alpha-tocopherol phosphate	0.00003
d-biotin	0.00004
Dexpanthenol	0.00008
choline chloride	0.00360
folic acid	0.00002
i-inositol	0.00028
menadione	0.00006
niacin	0.00024
niacinamide	0.00034
para-aminobenzoic acid	0.00036
pyridoxal HCl	0.00015
pyridoxine HCl	0.00023
riboflavin	0.00003
Vitamin A acetate (retinol)	0.00059

Component	Preferred Concentration (mM)
Vitamin D (calciferol (ergo))	0.00054
<b>Other Additives</b>	
D-glucose	4.15900
adenine sulfate	0.04300
adenosine 5-triphosphate	0.00190
adenosine 5-phosphate	0.00073
cholesterol	0.00052
deoxyribose	0.00370
glutathione	0.00016
guanine HCl	0.00160
HEPES	25.00000
hypoxanthine sodium	0.00290
D-ribose	0.00330
Thymine	0.00240
tween 80	0.01500
Uracil	0.00240
xanthine Na	0.00034

Advantageously, aminoguanidine may be added to the cell culture medium of the present invention. Aminoguanidine is an L-arginine analogue and acts as a nitric oxide inhibitor. Nitric oxide and its metabolites are known to cause cellular death from nuclear destruction and related injuries. Other L-arginine analogues, such as N-monomethyl L-arginine, N-nitro-L-arginine or D-arginine could also be used in the present invention. Aminoguanidine is provided at a concentration of about 15 to about 250  $\mu$ M, preferably about 30 to 180  $\mu$ M, most preferably about 80 to about 120  $\mu$ M. In one embodiment, the concentration of aminoguanidine is about 100  $\mu$ M.

The concentration of L-cysteine is also increased in the cell culture

medium of the present invention. L-cysteine acts as a scavenger of already formed nitric oxide and thereby prevents nitric oxide induced cellular damage. Additionally, L-cysteine may obscure immune recognition sites on the cultured cells by sulfhydryl bond formation to integral surface proteins containing sulfur groups. Further, L-cysteine provides sulfhydryl bonds which strengthen cell membranes. The preferred final concentration of L-cysteine is about 50 to about 300  $\mu\text{M}$ , preferably about 80 to about 250  $\mu\text{M}$ , most preferably about 150 to about 200  $\mu\text{M}$ . In one embodiment, the final concentration is about 180  $\mu\text{M}$ .

Although it is possible to use the cell culture medium of the present invention as a serum-free medium, albumin or other nutrient sources may be added. Use of albumin instead of conventional sera reduces cost and facilitates transplantation of cells. It is recognized that any source of albumin may be used and generally human albumin is used in most conventional culture media. For purposes of the present invention, the albumin or serum used is preferably isolated from the same species as the cells to be stored in the culture medium. For instance, for culturing of porcine pancreatic islet cells, porcine albumin or serum would be used. Use of albumin from the same species as the cultured cells negates the problems of cross-species antibody attacks upon the cells and IgM cross-linking. The cultured cells are more robust when same-species sera is used. Preferably, the concentration of albumin is about 5 to about 50  $\mu\text{l/ml}$ , preferably about 10 to about 30  $\mu\text{l/ml}$ , most preferably about 15  $\mu\text{l/ml}$  to about 25  $\mu\text{l/ml}$ . In one embodiment, the concentration of albumin is about 20  $\mu\text{l/ml}$ .

Other additives known in the art may also be added to the culture medium without departing from the present invention. For instance, antibiotics are preferably added to the medium. Any antibiotic known in the art may be used. It is recognized that the antibiotic of choice may vary depending on the type of cells. Preferred antibiotics include Coly-mycin, Amphotericin b, Ciprofloxacin and Gentamicin Sulfate and the like. The cell

culture medium may also be supplemented with additional L-glutamine to compensate for the degradation of that amino acid that may occur over time.

Table 2 below lists the particularly preferred additives and supplemented ingredients for one embodiment of the culture medium of the present invention and summarizes the final concentration ranges and preferred final concentrations for each ingredient.

Table 2

10

Components	Concentration Range	Preferred Concentration
Albumin	5-50 $\mu$ l/ml	20 $\mu$ l/ml
L-Cysteine HCl	50-300 $\mu$ M	180 $\mu$ M
Aminoguanidine	15-250 $\mu$ M	100 $\mu$ M
Coly-Mycin	5-20 $\mu$ g/ml	10 $\mu$ g/ml
Amphotericin B	2-6 $\mu$ M	3.382 $\mu$ M
Ciprofloxacin	2-6 $\mu$ g/ml	5 $\mu$ g/ml
Gentamicin Sulfate	2-6 $\mu$ g/ml	4.8 $\mu$ g/ml
L-Glutamine	5-15 $\mu$ M	10 $\mu$ M
L-Glutamic Acid	2-60mM	15mM
L-arginine HCl	2-60mM	10mM

In another embodiment of the cell culture medium of the present invention, the medium comprises at least one nitric oxide inhibitor. The nitric oxide inhibitors are present in an amount of about 15 to about 600  $\mu$ M, preferably about 100 to about 500  $\mu$ M, more preferably about 200 to about 350  $\mu$ M. Nitric oxide inhibitor is broadly defined as including any composition or agent that inhibits the production of nitric oxide or scavenges or removes existing nitric oxide. Nitric oxide is a pleiotropic mediator of inflammation. Nitric oxide is a soluble gas produced by endothelial cells,

macrophages, and specific neurons in the brain, and is active in inducing an inflammatory response. Nitric oxide and its metabolites are known to cause cellular death from nuclear destruction and related injuries. Suitable nitric oxide inhibitors for the buffer medium include, but are not limited to, L-cysteine, L-arginine analogues, cystine, and heparin. Aminoguanidine is a preferred L-arginine analogue. Other L-arginine analogues, such as N-monomethyl L-arginine, N-nitro-L-arginine or D-arginine may also be used in the present invention. Since nitric oxide is generally produced when cells are experiencing stress, such as trauma caused by enzymatic digestion, this embodiment of the cell culture medium is useful in preventing cellular death during periods of cell stress, even without the addition of supplemental amounts of polar amino acids.

In a preferred embodiment, aminoguanidine is provided at a concentration of about 15 to about 250  $\mu\text{M}$ , preferably about 30 to 180  $\mu\text{M}$ , most preferably about 80 to about 120  $\mu\text{M}$ . In one embodiment, the concentration of aminoguanidine is about 100  $\mu\text{M}$ . This embodiment of the medium preferably also includes L-cysteine. The final concentration of L-cysteine may range from about 50 to about 300  $\mu\text{M}$ , preferably about 80 to about 250  $\mu\text{M}$ , most preferably about 150 to about 200  $\mu\text{M}$ . In one embodiment, the final concentration is about 180  $\mu\text{M}$ .

Additionally, this embodiment of the culture medium may include a superoxide inhibitor. A preferred superoxide inhibitor is ethylenediaminetetraacetic acid (EDTA). Superoxide is a highly toxic reactive oxygen species, whose formation is catalyzed by divalent transition metals, such as iron, manganese, cobalt, and sometimes calcium. Highly reactive oxygen species such as superoxide ( $\text{O}_2^-$ ) can be further converted to the highly toxic hydroxyl radical ( $\text{OH}^\cdot$ ) in the presence of iron. By chelating these metal catalysts, EDTA serves as an antioxidant. The concentration range for the superoxide inhibitor is about 0 to about 10 mM, preferably 1 to about 8 mM, most preferably about 2 to about 6 mM. In a preferred embodiment, the



superoxide inhibitor is present at a concentration of about 4 mM.

### Matrix

The present invention also provides a hydrogel matrix for storage and  
5 transplantation of cells. The matrix is suitable for use with a variety of cells  
including cells derived from tissue of the lung, liver, kidney, thymus, thyroid,  
heart, brain, pancreas, and the like. The matrix of the present invention  
provides numerous advantages over matrixes of the prior art. The matrix of  
the present invention is able to sustain cells and complex clusters of cells such  
10 as islets. One advantage of the matrix is its ability to immobilize water at  
appropriate storage temperatures and provide binding sites for cells that  
apparently stimulate growth in terminal cell types, such as beta cells.

The matrix of the present invention also contains materials that provide  
scaffolding for both cellular attachment and protection. This attribute of the  
15 matrix obviates the need for sera in maintaining long term cell cultures, such  
as long term cultures of islets, pancreatic acinar tissue, hepatocytes, and  
erythrocytes. The matrix may be mixed with cells to form a transplant for  
injection into a host organism at a transplant site without the use of an  
additional protective carrier device. Transplant site is intended to mean the  
20 predetermined site where the transplant will be placed within the host  
organism. In this manner, the matrix allows transplantation of cells through a  
non-invasive and simple procedure.

A surprising feature of the matrix of the present invention is that use of  
the matrix allows transplantation of pancreatic islet cells at lower purity levels.  
25 Conventionally, islet cells are utilized at high purity levels to avoid substantial  
amounts of acinar cells in contact with the islet cells because of digestion of  
the islet cells by the acinar cells' digestive enzymes. This results in very  
costly and time consuming purification methods, as well as disposal of most  
pancreatic tissue because of the presence of acinar tissue. The matrix of the  
30 present invention allows coexistence of acinar cells with islet cells *in vitro* and

after transplantation in the host organism. The matrix allows the use of cell mixtures containing as much as 70% by volume or more of acinar cells. It is believed that the optimum range is about 30 to about 40% by volume islet purity.

5           The unpurified pancreatic tissue also functions better than purified islet cells. Unpurified pancreatic tissue has been shown to exhibit insulin pulsatility that more closely simulates the insulin pulsatility seen in the normal functioning of pancreatic tissue of a non-diabetic organism. The advantage of using unpurified cells is their ability to mimic normal pancreatic functions,  
10       such as insulin pulsatility. The insulin pulsatility of normally functioning pancreatic tissue is characterized by peak concentrations occurring every 5-10 minutes.

          Another feature of the matrix is its ability to stimulate or enhance vascularization in surrounding tissue. Vascularization refers to the formation  
15       of blood vessels. Stimulation or enhancement of vascularization is defined as increasing blood vessel formation and resulting blood circulation beyond that which would occur naturally. Due to the vascularization effect, an effective amount of the matrix may be applied to a transplant site prior to the transplant. An effective amount is an amount necessary to stimulate the flow  
20       of blood to the transplant site. In this manner, the matrix improves vascularization at the transplant site so that a blood supply is already available for the cells when the transplant occurs. However, matrix is routinely applied to the transplant site at the time of the procedure with neovascularization occurring within 4 to 7 days. The vascularization effect of the matrix  
25       increases the likelihood of long-term cell viability in a host organism.

          It is also recognized that the matrix may be used to treat conditions benefited by increased vascularization. Such conditions include those which would benefit from an increased supply of blood such as gangrene, wound sites, and general poor circulation problems. Additionally, formation of new  
30       blood vessels in the heart is critically important in protecting the myocardium

from the consequences of coronary obstruction. Injection of the matrix into ischemic myocardium may enhance the development of collaterals, accelerate the healing of necrotic tissue and prevent infarct expansion and cardiac dilatation.

5           The matrix is suitable for use in the transplantation of cells within a transplant device such as described in U.S. Patent Application Serial No. 08/568,694, which is herein incorporated by reference in its entirety. A transplant device is any device designed to contain and protect cells  
10       transplanted into a host organism for the production of hormones or other factors. Examples of other transplant devices suitable for use with the matrix include those described in U.S. Patent Nos. 5,686,091, 5,676,943 and 5,550,050. However, as discussed above, the matrix may be used as the sole transplant vehicle without using such devices.

          The matrix also finds use in storage of cells without loss of viability or  
15       specialized cell function. For long term storage, cells may be frozen in the matrix without significant loss of viability. This has application in shipping blood cells, hepatocytes, pancreatic tissue, hemopoietic stem cells, bone marrow, Leydig cells, thyroid cells, pituitary cells, cardiac cells, renal cells, and others either alone or in combination, for clinical or research applications.

20           Current blood banking techniques allow erythrocytes to be stored for only two months. The matrix of the present invention allows erythrocytes to remain morphologically intact for seven months. The matrix has also been demonstrated to maintain the highly specialized function of cells for extended periods of time. Hepatocytes have maintained their specialized thiol  
25       transferase, albumin, and cytochrome p450 enzymes for up to 8 weeks *in vitro* when stored in the matrix. Drug metabolizing activity has been maintained for at least two weeks during storage of hepatocytes in matrix. Human red blood cells have been stored for over 8 months and reconstituted by adding water without cellular lysis. A human neuron cell line has been demonstrated  
30       to keep specific message for up to 4 weeks. The matrix thus appears to be

able to keep a variety of partially or totally isolated cells alive and functional for extended periods of time.

An important feature of the matrix of the present invention is the increased level of polar amino acid groups. The addition of polar amino acids  
5 increases the number of hydrogen bonding moieties which subsequently increase the rigidity of the matrix. The increased hydrogen bonding attracts and immobilizes water. This immobilization of water reduces cell membrane damage caused by temperature changes. It is also believed that the polar amino acid groups contribute to molecular encapsulation of the cells therein  
10 and block the immune recognition sites present on the cell surface. This characteristic allows cells stored in the matrix to be directly injected into a host organism without recognition by the host organism's immune system that the injected cells are foreign. This would allow cross-species transplantation of cells without immunosuppression. For example, porcine pancreatic islet  
15 cells could be injected into human hosts using the matrix of the present invention. Use of the matrix of the present invention obviates the need for additional protective measures to prevent a negative immune system response by the host organism.

The matrix may contain an effective amount of polar amino acids  
20 therein. The polar amino acids may be selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid, or other amino acids or other polar chemicals. An effective amount is the amount necessary to increase the rigidity of the matrix and allow direct injection of the matrix with cells encapsulated therein into a host organism without  
25 immunosuppression. In one embodiment, the concentration of polar amino acids is raised to a final concentration of between about 3 to about 150 mM, preferably about 10 to about 65 mM, and more preferably about 15 to about 40 mM.

Advantageously, the added polar amino acids comprise L-glutamic  
30 acid, L-lysine, and L-arginine. The final concentration of L-glutamic acid is

about 2 to about 60 mM, preferably about 5 to about 40 mM, most preferably about 10 to about 20 mM. In one embodiment, the concentration of L-glutamic acid is about 15 mM. The final concentration of L-lysine is about 0.5 to about 30 mM, preferably about 1 to about 15 mM, most preferably about 1 to about 10 mM. In one embodiment, the concentration of L-lysine is about 5.0 mM. The final concentration of L-arginine is about 1 to about 40 mM, preferably about 1 to about 30, most preferably about 5 to about 15 mM. In one embodiment, the final concentration of L-arginine is about 10 mM.

The matrix of the present invention is a combination of a gelatin component and a liquid composition. The gelatin acts as a substrate for cellular attachment. The preferred gelatin component is denatured collagen. Denatured collagen contains polar and non-polar amino acids that readily form a gel based on amine, carboxyl group, hydroxyl group, and sulfhydryl group interactions. The matrix is designed to be in a free flowing or liquid phase at host body temperature in order to provide maximum diffusion across the membrane *in vivo*. The matrix remains in solid phase at the lower storage temperatures, such as 4°C.

Boiling or otherwise treating intact collagen to form denatured collagen breaks covalent chemical bonds and increases the number of heat sensitive hydrogen bonds and dipole moment attractions. By replacing the covalent chemical bonds with temperature sensitive bonds and attractions, the desired cells can be embedded in a solid matrix formulation at colder temperatures for sustained storage. Boiling or otherwise treating intact collagen breaks the tightly coiled helical tropocollagen subunits and causes the subunits to open up into separate peptide chains. These uncoiled strands provide multiple binding areas for cells to attach.

The gelatin is present at a concentration of about 0.01 to about 40 mM, preferably about 0.05 to about 30 mM, most preferably about 1 to 5 mM. Advantageously, the gelatin concentration is approximately 1.6 mM. The above concentrations provide a solid phase at storage temperature and a liquid

phase at transplant temperature.

The gelatin component of the matrix of the present invention is mixed with a liquid composition. The liquid composition is preferably based upon a standard culture medium, such as Medium 199, supplemented with additives  
5 and additional amounts of some medium components, such as supplemental amounts of polar amino acids as described above.

An additional amount of L-cysteine may be added to the matrix of the present invention. L-cysteine acts as a nitric oxide scavenger and obscures immune recognition sites on the surface of the cells. L-cysteine also provides  
10 disulfide linkages which increases the matrix's resistance to force and further protects the cells contained therein. The final concentration of L-cysteine is about 5 to about 500  $\mu\text{M}$ , preferably about 10 to about 100  $\mu\text{M}$ , most preferably about 15 to about 25  $\mu\text{M}$ . In one embodiment, the final concentration is about 20  $\mu\text{M}$ .

Advantageously, aminoguanidine is also added to the matrix of the present invention. As indicated above, aminoguanidine is an L-arginine  
15 analogue and acts as a nitric oxide inhibitor. Other L-arginine analogues could also be used in the present invention. The final concentration of aminoguanidine is about 5 to about 500  $\mu\text{M}$ , preferably about 10 to about 100  
20  $\mu\text{M}$ , most preferably about 15 to about 25  $\mu\text{M}$ . In one embodiment, the final concentration is about 20  $\mu\text{M}$ .

In order to increase cell binding, intact collagen may be added in small amounts to provide an additional binding network for the cells contained in the matrix. The final concentration of intact collagen is from about 0 to about 5  
25 mM, preferably 0 to about 2 mM, most preferably about 0.05 to about 0.5 mM. In one embodiment, the concentration of intact collagen is about 0.11 mM.

Additionally, the matrix to the present invention may include a divalent chelator which increases the rigidity of the matrix by removing inhibition of -  
30  $\text{NH}_2$  to  $-\text{COOH}$  hydrogen bonding. The divalent chelator also protects against

microbial contamination of the matrix. A preferred divalent chelator is EDTA. The concentration range for the chelator is about 0 to about 10 mM, preferably 1 to about 8 mM, most preferably about 2 to about 6 mM. In a preferred embodiment, EDTA is present at a concentration of about 4 mM.

- 5 Conventional antibiotics can also be added to further protect against microbial contamination.

- As indicated above, the matrix of the present invention does not require the presence of sera in order to maintain long term cell cultures. However, albumin or other nutrient sources may be added to the matrix of the
- 10 present invention if desired. Preferably, the albumin used is of the same species as the cells contained within the matrix. As described above, use of the same species albumin promotes increased robustness in the cells contained in the matrix. The concentration of albumin is about 0 to about 2% by volume, preferably 0 to about 0.5% by volume, most preferably about 0 to
- 15 about 0.1% by volume. In a preferred embodiment, the concentration of albumin is about 0.05% by volume.

- The addition of high concentrations of polar amino acid enhancements, or other polar substrates, further improves the immobilization of water such that cells or cell combinations may be frozen to at least -20°C without
- 20 apparent morphologic or functional damage. The increased concentrations of L-glutamic acid, L-lysine, L-arginine, in addition to increased concentrations of cysteine, result in increased denatured connective tissue immobilization of water at cold temperatures. Thus, the current invention demonstrates a long term cryopreservation ability without the use of membrane solubilizing agents
- 25 such as DMSO (Dimethyl Sulfoxide) that are commonly used to cryopreserve isolated cells.

- For long term storage, an effective amount of cryoprotectant may be added that allows the matrix to be stored at lower temperatures without cellular damage. Preferably, the cryoprotectant is metabolically stable and
- 30 capable of creating an inert cushion to prevent thermal expansion and

contraction of cells. A preferred cryoprotectant is sulfated dextran. The cryoprotectant is present at a concentration of about 0 to about 2 mM, preferably 0 to about 1 mM, most preferably about 0 to about 0.1 mM. In one embodiment, the cryoprotectant is present in a concentration of about 5 0.086 mM.

Table 3 below lists particularly preferred key components of the matrix of the present invention along with suitable concentrations as well as preferred concentrations for each component.

10

Table 3

Component	Concentration Range	Preferred Concentration
L-glutamic acid	2 to 60 mM	15 mM
L-lysine	.5 to 30 mM	5.0 mM
L-arginine	1 to 40	10 mM
Gelatin	0.01 to 40 mM	1.6 mM
L-cysteine	5 to 500 $\mu$ M	20 $\mu$ M
Aminoguanidine	5 to 500 $\mu$ M	20 $\mu$ M
Intact collagen	0 to 5 mM	0.11 mM
EDTA	0 to 10 mM	4 mM
Albumin	0 to 2% by volume	0.05% by volume
Dextran	0 to 2 mM	0.086 mM

Matrix Preparation

Place 835 ml of Medium 199 into a beaker. While stirring, heat the solution to 50°C. Using a syringe, add 20 ml of albumin to the stirred solution. Pipette 63.28  $\mu$ l of L-cysteine, 1 ml of L-glutamine and 200  $\mu$ l of aminoguanidine into the stirred beaker. Add the following gamma irradiated dry raw materials: 120 grams of denatured collagen, 50 grams of dextran, and 0.1 grams of intact collagen. Use a glass stirring rod to aid mixing of the dry 15



materials into solution. Pipette 8 ml of EDTA into the solution. Pipette 5 ml of L-glutamic acid, 5 ml of L-lysine acetate, and 5 ml of L-arginine HCl into the stirred beaker. Note that the solution will turn yellow. Use 10% NaOH to adjust the pH of the matrix solution to a final pH of  $7.40 \pm 0.05$ .

- 5           Cells may be embedded in the matrix of the present invention using the following procedure. Aspirate the supernatant from centrifuged cell pellets. Add a volume of cell culture medium and matrix to the cell pellets. Add a volume of matrix approximately equal to about 4 times the pellet volume. Add a volume of cell culture medium to the cell pellets equaling
- 10           approximately 0.05 times the matrix volume added. Store the encapsulated cells at refrigerated temperatures if not using immediately.

The present invention also provides a method of refeeding cells stored in the matrix of the present invention. Conventionally, cell cultures could not be maintained for a duration long enough to require refeeding of the cells.

- 15           However, using the matrix of the present invention, cell viability may be maintained for longer periods of time, necessitating periodic refeeding of the cells. Additionally, bringing the cells to room temperature periodically allows evaluation of cell function and viability and encourages the development of communication networks between cells.

- 20           Periodically, during refrigeration of the cell/matrix mixture, the cells may be refed or metabolically "walked" using the following procedure. First the stored cell/matrix mixture is retrieved from refrigeration. The mixture is examined for excess fluid. If excess fluid is present, the fluid is pipetted away and discarded. Cell culture medium is then added to the mixture. In one
- 25           embodiment, 400  $\mu$ l of the cell culture medium of the present invention is pipetted into each 15 ml container of the cell/matrix mixture. The container is shaken to distribute the cell culture medium over the entire cell/matrix mixture. The container is then capped and transferred to a 37°C incubator. The containers are incubated for about two hours and then transferred back to
- 30           refrigeration.

The following examples are offered by way of illustration and not by way of limitation.

### Experimental

#### Example 1

5           The bottom of Figure 1 lists the amount of porcine c-peptide in ng/ml per week produced by a rabbit designated Rabbit 6. Rabbit 6 was part of a study that utilized a bioartificial endocrine device containing porcine islets in the matrix of the present invention. The bioartificial device was taken out after 7 weeks, designated "Week 0". At that time the device was surgically  
10 removed, and it was discovered that the device had ruptured resulting in the porcine islets and matrix leaking into the surrounding tissue of Rabbit 6. However, as indicated in Figure 1, Rabbit 6 continued to produce detectable levels of porcine c-peptide until week 27. This suggests that the porcine islets in the matrix of the present invention produced a well vascularized,  
15 immunoprivileged site within the tissue of the rabbit.

#### Example 2

          Also referring to Figure 1, a rabbit designated as Rabbit 3 was injected with 3 ml of unpurified pancreatic tissue (7% islet tissue) and 2.6 ml of  
20 purified islet tissue (100% purity). Four weeks after the injection, the rabbit displayed a significant concentration of porcine c-peptide and achieved levels of up to 0.3 ng/ml during IV glucose tolerance testing 6 weeks after the injection. The rabbit continued to produce porcine c-peptide for 13 weeks after the injection. This indicates that unpurified pancreatic tissue functions  
25 effectively *in vivo*.

#### Example 3

          Referring again to Figure 1, a non-diabetic dog was injected with unpurified islets (dog 22136). After injection, the dog produced 0.2 ng/ml  
30 porcine c-peptide within 6 days of implant and demonstrated levels of at least

0.1 ng/ml porcine c-peptide at all 15 blood draw points during a 90 minute IV glucose tolerance test. Two other dogs were injected with partially purified islets. Those two dogs demonstrated 0.1 ng/ml on one occasion each after 4 weeks.

5           This supports a finding that unpurified pancreatic tissue, after being placed in the matrix of the present invention, produces mature insulin product as measured by c-peptide much sooner and in much greater quantity per islet than purified islets. This is an unexpected finding because previous findings indicated that islet transplantation required use of substantially purified islets  
10 to prevent digestion of the islets by the acinar cells. However, using the matrix of the present invention, islets coexist with acinar cells without negative effect and appear to regain physiological function faster, both in terms of quantity produced and insulin pulsatility.

15   Example 4

Referring to Figure 2, three semipermeable devices consisting of the same number of porcine islets in enhanced matrix (containing polar amino acids), but differing amounts of acinar tissue were perfused for 115 minutes at 37°C with a physiologic buffer containing 100 mg/dl glucose. At 115  
20 minutes, a bolus of glucose was injected into the solution to bring the total glucose concentration up to 300 mg/dl for a period of 40-60 minutes post-bolus.

The highly purified islet device (95% islet purity) released 11,000 uU insulin/ml within 5 minutes of seeing the glucose bolus, but only had one  
25 much smaller insulin peak of 5000 uU insulin/ml about 25 minutes later. Slightly less purified material (85% islet purity) demonstrated the same initial peak of insulin at 5 minutes post bolus of 11,000 uU insulin/ml, but showed another peak of 8,000 uU insulin/ml 15 minutes post bolus, followed by small pulsations 25-45 minutes post bolus. In the device containing only 50% islets,  
30 there were 4 peaks over a 22 minute period of between 11,000 and 12,000 uU

insulin/ml demonstrating physiologic insulin pulsatility. These data demonstrate the ability of the matrix to sustain partially purified pancreatic tissue and allow such tissue to function physiologically.

#### 5 Example 5

Figure 3 illustrates the amount of insulin collected as a function of number of islets, purity and age. With 38% purity, over two hours with only 9.2 thousand islets, 3,550,000 uU insulin (or 3.65 units) were produced. When the number of islets were doubled to 18.4 thousand islets, the total  
10 insulin doubled to 8,400,000 uU insulin produced in 2 hours. Similar results, though slightly lower, were obtained from islets of only 31% purity. Of note is the extremely small volume of tissue in the matrix required to produce such large amounts of insulin -- only 0.2 or 0.4 ml. The apparent optimal range is from about 30 to about 40% islet purity.

15 The matrix thus has been demonstrated to improve the communication among different cell types that apparently results in a substantial improvement of function. Thus the matrix not only protects the cells from physical and immunologic trauma, it also facilitates cellular communication *in vitro* so that the cells can maintain their function as seen *in vivo*.

20

#### Example 6

Two canine subjects were pancreatectomized within two weeks of each other and were treated with injections of mixtures of Ultralente and Regular insulin twice daily. Both animals were fed identical amounts of food with  
25 Viokase added to replace pancreatic digestive enzymes. Blood glucose values were determined in the morning and late afternoon, and exogenous insulin requirements were based on these values.

For four weeks prior to one of the dogs being injected with 8 cc of one volume unpurified pancreatic tissue per four volumes matrix, the two dogs had  
30 statistically equivalent blood glucose determinations, and received the same

dose of insulin twice daily. The blood glucose levels of a dog that was not injected (darkly shaded line) and a dog that was ultimately injected intramuscularly on Day 0 (lightly shaded line) are shown in Figure 4. The daily AM and PM blood glucose determinations are shown beginning one week prior to injection, and out for a total of three weeks (22 days).

Figure 4 demonstrates that there was no statistically significant difference in the AM or PM blood glucose determinations during the week prior to one dog receiving the porcine tissue injection. Beginning the day of injection, the injected dog had a statistically significant decrease in the PM blood glucose on the same insulin dose as the uninjected dog. There was no statistical difference in the AM blood glucose during the first week after injection, probably reflecting the increased insulin resistance that mammals experience in the morning due to the effects of counter regulatory hormones such as cortisol and growth hormone. Type I diabetics generally require twice as much insulin in the AM to cover the same ingestion of carbohydrates as they require pre-supper because of this AM "cortisol" effect.

Beginning seven days after the injection, blood glucose levels in the injected dog clearly separated from those of the uninjected animal. Both animals had their insulin decreased 15% beginning week two. The injected dog's glucose continued to normalize, while the uninjected dog's blood glucose rose as expected. The injected animal continued to have statistically significant decreased blood glucose compared to the uninjected animal over the three week period. At that point, we separated the animals' insulin dose so that the uninjected dog could be better controlled.

25

#### Example 7

The uninjected animal in Example 6 was injected with unpurified porcine pancreatic material embedded in the matrix of the present invention to further protect the cells from immune recognition. Approximately 8 cc of this material was injected intramuscularly into the previously uninjected dog.

30

Beginning that evening, the dog's blood glucose fell, and the total insulin dose was cut 33%. The dog went at least seven days with the change in daily mean blood glucose and daily mean insulin dose shown below:

5

Table 4

	Mean Daily Blood Glucose	Mean total daily insulin dose
Seven days prior to injection	180 mg%	64 units
Seven days post injection	101 mg%	43 units

These data demonstrate that the injected porcine tissue has the effect of more than 20 units of exogenously administered insulin, since the average blood glucose has fallen nearly 40% and normalized on 20 units less insulin.

10 The total daily insulin released in the average human subject is approximately 0.25 units/kg body weight, or 20 units per day in an 80 kg man. These data do not necessarily reflect 20 units of insulin production, since the pulsatile release of the pancreatic tissue probably increases the animal's insulin sensitivity.

15 These data clearly show the ability of unpurified porcine pancreatic tissue to function without the use of immunosuppression. Based on the above figures, isolated cells from three pancreases could treat 30-50 patients.

#### Example 8

20 Islet beta cells in the matrix of the present invention were observed after 7 days at 4°C in the presence of a large acinar cell with digestive granules present. The cells appeared to have normal cytoplasm and intact ultrastructure, compared to pancreatic cells kept in Medium 199 under the same conditions. The islet cells in Medium 199 showed their cytoplasm  
 25 washed out with the acinar cell releasing digestive enzyme material.

#### Example 9

Porcine liver was digested by dicing the organ into small slices, and placing the material in collagenase for five minutes. The digested hepatocytes, Kupfer cells, and epithelial cells were then placed in the above matrix and kept for 10 days at 4°C. Trypan blue exclusion stain revealed  
5 90% viability at 10 days. In another experiment, gene expression for albumin was measured in 77 day old cells and lidocaine metabolism measured in 13 day old porcine hepatocytes in matrix of the present invention.

#### Example 10

10 Fresh whole blood from an adult male donor was centrifuged, and serum removed. The cellular pellet was divided into two 1 ml aliquots, and placed in either 4 ml Hanks Buffered Saline Solution or the above matrix, and stored at 4°C for seven months.

At the end of seven months, the cells stored in the Hanks Solution had  
15 totally lysed, with no cells seen under 100 X light microscopy. The matrix-containing cellular pellet was heated to 37°C, and diluted 1:1 with Hanks Solution. Intact erythrocytes with biconcave morphology at 100X light microscopy were present in the matrix-containing pellet.

#### 20 Example 11

Unpurified and purified porcine pancreatic tissue was digested from fresh pancreata using standard collagenase digestion techniques. The unpurified or gradient purified samples were placed in a matrix containing 5 mM lysine, 5 mM arginine, and 10 mM glutamic acid, in addition to 180 µM  
25 cysteine, in a one part tissue volume to four parts matrix volume, placed in polypropylene tubes, and stored from 1 day to 6 weeks at -20°C. The previously frozen tissue was then thawed and stained with TSQ (N-6-methyl-8-quinolyl paratoluenesulfonamide), a fluorescent zinc dye that indicates intracellular presence of insulin.

30 Inspection of the cells indicated appropriate morphology of both the

islet tissue and digestive acinar cells in an unpurified preparation that was frozen for 6 weeks.

Many modifications and other embodiments of the invention will come to mind to one skilled in the art to which this invention pertains having the  
5 benefit of the teachings presented in the foregoing descriptions and associated drawings. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a  
10 generic and descriptive sense only and not for purposes of limitation.



## THAT WHICH IS CLAIMED:

1. A cell culture medium for long-term storage and proliferation of cells, comprising:  
an effective amount of polar amino acids, said polar amino acids  
5 selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid.
2. A cell culture medium according to Claim 1, wherein said effective amount comprises about 5 to about 150 mM of polar amino acids.  
10
3. A cell culture medium according to Claim 2, wherein said effective amount comprises about 10 to about 65 mM of polar amino acids.
4. A cell culture medium according to Claim 1, wherein said polar amino  
15 acids are selected from the group consisting of arginine, glutamic acid or mixtures thereof.
5. A cell culture medium according to Claim 4, wherein said polar amino acids comprise about 2 to about 60 mM of L-arginine and about 2 to about 60  
20 mM of L-glutamic acid.
6. A cell culture medium according to Claim 5, wherein said polar amino acids comprise about 5 to about 30 mM of L-arginine and about 5 to about 30 mM of L-glutamic acid.  
25
7. A cell culture medium according to Claim 6, wherein said polar amino acids comprise about 10 mM of L-arginine and about 15 mM of L-glutamic acid.
- 30 8. A cell culture medium according to Claim 1, further comprising cells,

wherein said cells are selected from the group consisting of lung cells, liver cells, kidney cells, thymus cells, thyroid cells, heart cells, brain cells, pancreatic islet cells, pancreatic acinar cells and mixtures thereof.

- 5     9.     A cell culture medium according to Claim 1, further comprising about 50 to about 300  $\mu$ M of L-Cysteine.
10.     A cell culture medium according to Claim 1, further comprising about 15 to about 250  $\mu$ M of an L-arginine analogue.
- 10     11.     A cell culture medium according to Claim 1, further comprising about 5 to about 50  $\mu$ g/ml of a nutrient source selected from the group consisting of albumin and serum.
- 15     12.     A cell culture medium according to Claim 11, wherein said nutrient source is isolated from the same species as the cells.
13.     A cell culture medium, comprising:  
         at least one nitric oxide inhibitor, said at least one nitric oxide inhibitor  
20     present in an amount of about 15 to about 600  $\mu$ M.
14.     A cell culture medium according to Claim 13, wherein said at least one nitric oxide inhibitor is present in an amount of about 200 to about 350  $\mu$ M.
- 25     15.     A cell culture medium according to Claim 13, wherein said at least one nitric oxide inhibitor is selected from the group consisting of L-cysteine, L-arginine analogues, cystine, and heparin.
16.     A cell culture medium according to Claim 13, wherein said at least one  
30     nitric oxide inhibitor comprises about 15 to about 250  $\mu$ M of aminoguanidine

and about 50 to about 300  $\mu$ M of L-cysteine.

17. A cell culture medium according to Claim 16, wherein said at least one nitric oxide inhibitor comprises about 80 to about 120  $\mu$ M of aminoguanidine  
5 and about 150 to about 200  $\mu$ M of L-cysteine.

18. A cell culture medium according to Claim 13, further comprising a superoxide inhibitor.

- 10 19. A cell culture medium according to Claim 13, wherein said superoxide inhibitor is present in an amount of about 1 to about 8 mM.

20. A cell culture medium according to Claim 18, wherein said superoxide inhibitor is EDTA.

15

21. A hydrogel matrix for long-term storage and proliferation of cellular tissue, comprising:

about 0.01 to about 40 mM of gelatin; and

an effective amount of polar amino acids, said polar amino acids

- 20 selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid.

22. A hydrogel matrix according to Claim 21, wherein said effective amount comprises about 3 to about 150 mM of polar amino acids.

25

23. A hydrogel matrix according to Claim 21, wherein said effective amount comprises about 10 to about 65 mM of polar amino acids.

24. A hydrogel matrix according to Claim 21, wherein said polar amino  
30 acids are selected from the group consisting of arginine, glutamic acid, lysine

and mixtures thereof.

25. A hydrogel matrix according to Claim 24, comprising:  
about 2 to about 60 mM of L-glutamic acid;  
5 about .5 to about 10 mM of L-lysine; and  
about 1 to about 40 mM of L-arginine.
26. A hydrogel matrix according to Claim 25, comprising:  
about 5 to about 40 mM of L-glutamic acid;  
10 about 1 to about 15 mM of L-lysine; and  
about 1 to about 30 mM of L-arginine.
27. A hydrogel matrix according to Claim 21, further comprising about 5  
to about 500  $\mu$ M of L-cysteine.  
15
28. A hydrogel matrix according to Claim 21, further comprising about 5  
to about 500  $\mu$ M of an L-arginine analogue.
29. A hydrogel matrix according to Claim 27, wherein said matrix  
20 comprises about 0.02 mM of L-cysteine.
30. A hydrogel matrix according to Claim 28, wherein said matrix  
comprises about 0.02 mM of an L-arginine analogue.
- 25 31. A hydrogel matrix according to Claim 28, wherein said L-arginine  
analogue is aminoguanidine.
32. A hydrogel matrix according to Claim 21, wherein said matrix  
comprises about 15 mM of L-glutamic acid.  
30

33. A hydrogel matrix according to Claim 21, wherein said matrix comprises about 10 mM of L-arginine.
34. A hydrogel matrix according to Claim 21, wherein said matrix  
5 comprises about 5 mM of L-lysine.
35. A hydrogel matrix according to Claim 21, further comprising about 0 to about 10 mM of a divalent chelator.
- 10 36. A hydrogel matrix according to Claim 35, wherein said divalent chelator is EDTA.
37. A hydrogel matrix according to Claim 21, further comprising about 0 to about 2% by volume of a nutrient source selected from the group consisting  
15 of albumin and serum.
38. A hydrogel matrix according to Claim 37, wherein said nutrient source is isolated from the same species as the cells.
- 20 39. A hydrogel matrix according to Claim 21, further comprising about 0 to about 2 mM of a cryoprotectant.
40. A hydrogel matrix according to Claim 39, wherein said cryoprotectant comprises dextran.  
25
41. A hydrogel matrix according to Claim 21, further comprising about 0 to about 5 mM of intact collagen.
42. A hydrogel matrix according to Claim 21, wherein said gelatin is  
30 denatured collagen.

43. A transplant for implanting in a host organism comprising cells encapsulated by a matrix comprising an effective amount of polar amino acids, said polar amino acids selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid.
44. A transplant according to Claim 43, wherein said effective amount comprises about 3 to about 150 mM of polar amino acids.
45. A transplant according to Claim 43, wherein said polar amino acids are selected from the group consisting of arginine, glutamic acid, lysine or mixtures thereof.
46. A transplant according to Claim 43, wherein said cells are selected from the group consisting of lung cells, liver cells, kidney cells, thymus cells, thyroid cells, heart cells, brain cells, pancreatic islet cells, pancreatic acinar cells and mixtures thereof.
47. A method for increasing insulin production in a transplant, said method comprising:
- providing a mixture of acinar cells and islet cells;
  - encapsulating the mixture in a matrix comprising an effective amount of polar amino acids to form a transplant, said polar amino acids selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid; and
  - injecting the transplant into a host organism.
48. A method according to Claim 47, wherein said effective amount comprises about 3 to about 150 mM of polar amino acids.

49. A method according to Claim 47, wherein said polar amino acids are selected from the group consisting of arginine, glutamic acid, lysine or mixtures thereof.
- 5 50. A method according to Claim 47, wherein the mixture of acinar cells and islet cells comprises at least about 30% by volume acinar cells.
51. A method according to Claim 47, wherein the mixture of acinar cells and islet cells comprises at least about 60% by volume acinar cells.
- 10 52. A method of metabolically refeeding stored cells, said method comprising:
- providing a container of stored cells at room temperature;
- adding cell culture medium to the container, said cell culture medium
- 15 comprising an effective amount of polar amino acids, said polar amino acids selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid; and
- incubating the container of stored cells.
- 20 53. A method according to Claim 52, wherein said effective amount of polar amino acids comprises about 3 to about 150 mM of polar amino acids.
54. A method according to Claim 52, wherein said polar amino acids are selected from the group consisting of arginine, glutamic acid, lysine or
- 25 mixtures thereof.
55. A method according to Claim 52, wherein said step of adding cell culture medium comprises adding about 10 to about 40  $\mu$ l/ml of stored cells.
- 30 56. A method of protecting cells during isolation of the cells after

enzymatic digestion of cell tissue, said method comprising:

collecting digestate from a digestion process; and

5 adding cell culture medium to the digestate, said cell culture medium comprising an effective amount of polar amino acids, said polar amino acids selected from the group consisting of arginine, lysine, histidine, glutamic acid,  
and aspartic acid.

10 57. A method according to Claim 56, wherein said effective amount of polar amino acids comprises about 3 to about 150 mM of polar amino acids.

58. A method according to Claim 56, wherein said polar amino acids are selected from the group consisting of arginine, glutamic acid, lysine or mixtures thereof.

15 59. A method of maintaining viability and functioning of cells during storage, comprising embedding the cells in a hydrogel matrix according to Claim 21.

20 60. A method according to Claim 59, wherein said cells are frozen during storage.



FIGURE 1

# Porcine C-Peptide after Injection of Porcine Tissue in Matrix

Week	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Rabbit 3	<0.1	0.2	0.2		0.1 0.2 (≡0-40) 0.3 (≡50,60)	<0.1	0.1	<0.1	0.1 (≡0,1)	<0.1	0.1	0.1	<0.1	necropsy
Dog 22136	<0.1	<0.1	0.2	<0.1, 0.1(≡60)	0.1(15 pls)	0.1(7pls) 0.2 (≡15)	0.1 (≡0-80) 0.2 (≡90)	pancreatectomy						

Week	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Rabbit 6	<0.1	<0.1	0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	0.1	0.2	0.1(≡0)	0.2	<0.1
		0.1												
	14	15	16	17	18*	19	20	21	22	23	24	25	26	27
	0.1	<0.1	0.1	<0.1	0.1	<0.1	<0.1	0.1	0.1, <0.1	<0.1	<0.1	<0.1	0.1	necropsy

# IN VITRO INSULIN PULSATILITY AS A FUNCTION OF ISLET PURITY

FIGURE 2

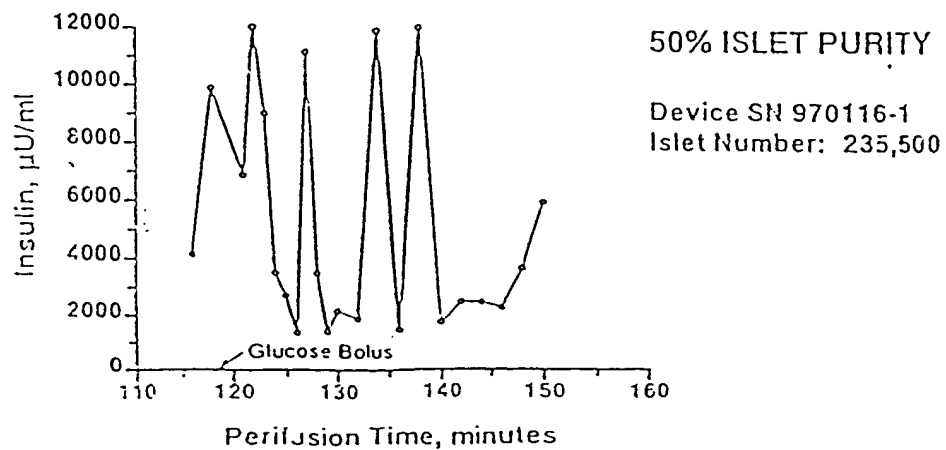
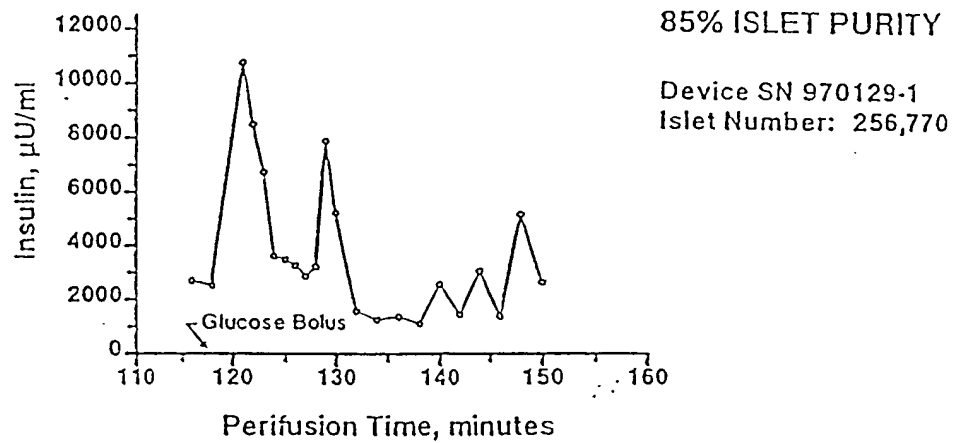
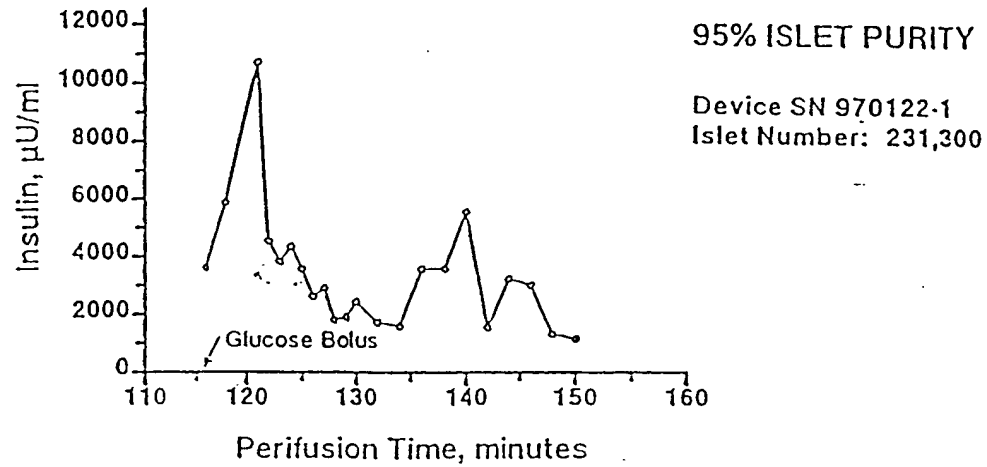


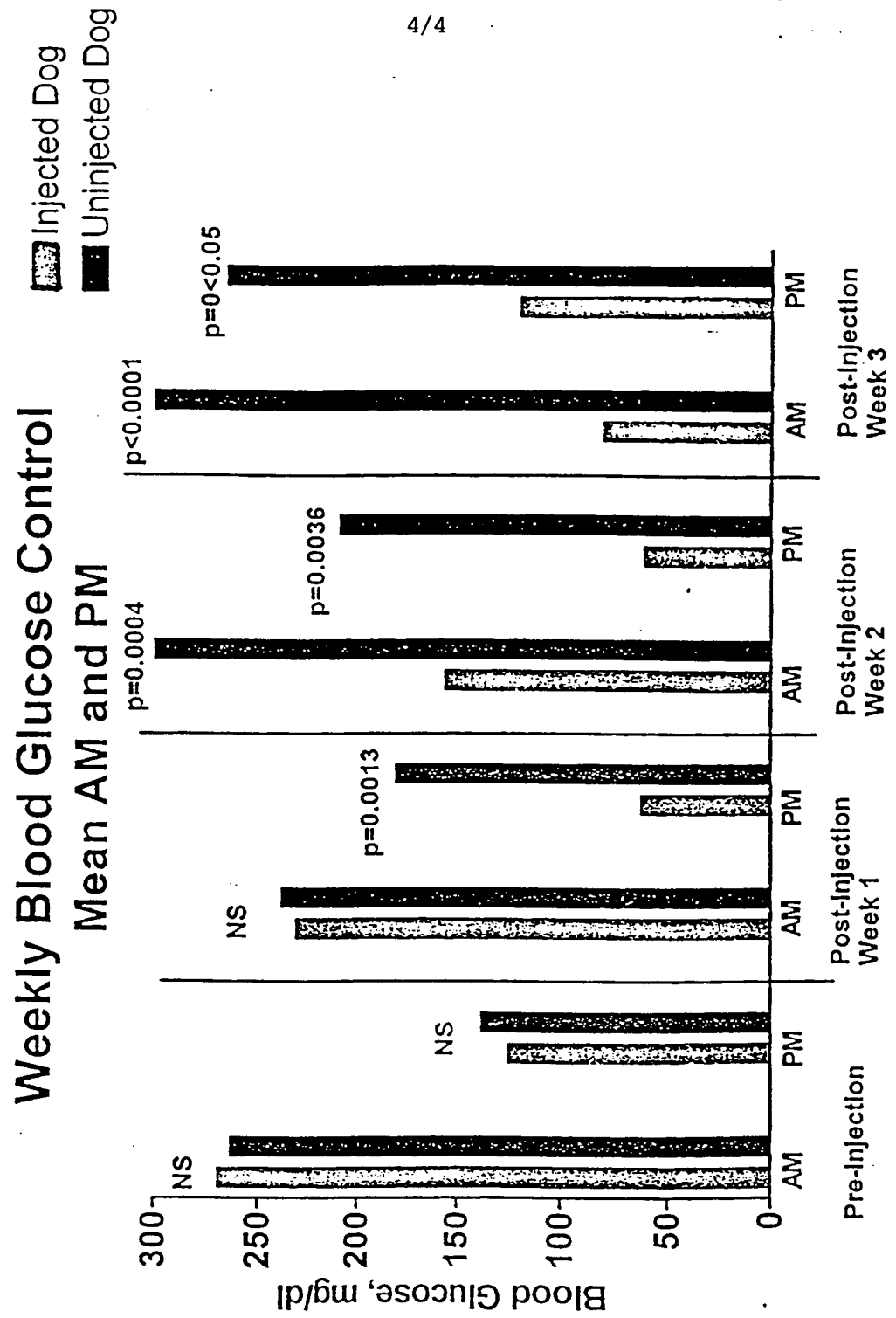
FIGURE 3

# Insulin Collection in Perifusion:

(as a function of islet number, purity and age)

Device Number	Performance in Perifusion		Device Characteristics			
	Insulin Collected (Units)	$\mu$ U/Islet Collected	Islet Age (Days)	# Islets (k EIN)	Islet/Matrix Volume (ml)	Islet Purity (%)
970404-1 -2	3.65	397	6	9.2	0.2	38
	3.5	380	6	9.2	0.2	38
-3 -9	8.42	458	6	18.4	0.4	38
	8.48	461	6	18.4	0.4	38
-5 -6	0.99	135	5	7.4	0.2	31
	0.81	110	5	7.4	0.2	31
-7 -8	2.64	180	5	14.7	0.4	31
	3.58	244	5	14.7	0.4	31

FIGURE 4





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 5/02, 5/06</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/02999</b> <b>(43) International Publication Date:</b> 20 January 2000 (20.01.00)
<b>(21) International Application Number:</b> PCT/US99/15464 <b>(22) International Filing Date:</b> 9 July 1999 (09.07.99) <b>(30) Priority Data:</b> 09/113,437      10 July 1998 (10.07.98)      US <b>(71) Applicant (for all designated States except US):</b> ENCELLE, INC. [US/US]; 2408 S. Charles Boulevard, Greenville, NC 27858 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> USALA, Anton-Lewis [US/US]; 237 Buckingham Drive, Winterville, NC 27890 (US). KLANN, Richard, Chris [US/US]; 239 East Main Street, Washington, NC 27889 (US). <b>(74) Agents:</b> HUMPHREY, Christopher, M. et al.; Alston & Bird LLP, P.O. Drawer 34009, Charlotte, NC 28234-4009 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK; LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 20 April 2000 (20.04.00)
<b>(54) Title:</b> MEDIUM AND MATRIX FOR LONG-TERM PROLIFERATION OF CELLS  <b>(57) Abstract</b>  <p>A cell culture medium and hydrogel matrix for long term storage and proliferation of cells is provided. The cell culture medium and hydrogel matrix may include an effective amount of polar amino acids, the polar amino acids selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid. One embodiment of the cell culture medium comprises about 5 to about 150 mM of polar amino acids. The hydrogel matrix comprises about 3 to about 150 mM of polar amino acids. L-arginine and L-glutamic acid are preferably supplemented in the cell culture medium. L-arginine, L-lysine, and L-glutamic acid are preferably supplemented in the hydrogel matrix. A method of maintaining viability and functioning of a transplant is also provided. The method of maintaining viability of a transplant includes encapsulating the cells in a hydrogel matrix and injecting the encapsulated cells into the host organism. The matrix of the present invention may also be used to promote vascularization in a transplant site prior to injection of cells.</p>		

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 99/15464

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12N5/02 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 16629 A (LIFE TECHNOLOGIES INC) 23 April 1998 (1998-04-23) the whole document ---	1-12, 52-58
X	WO 98 04681 A (BROWN MARIE E ; GENZYME CORP (US); HANLON JAMES G (US); YAEGER PETE) 5 February 1998 (1998-02-05) the whole document ---	1-12, 52-58
X	US 4 657 866 A (KUMAR SUDHIR) 14 April 1987 (1987-04-14) the whole document ---	1-12, 52-58
X	EP 0 481 791 A (WELLCOME FOUND) 22 April 1992 (1992-04-22)  the whole document ---	1-8, 10-12, 52-58
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*8\* document member of the same patent family

Date of the actual completion of the international search

27 January 2000

Date of mailing of the international search report

11.02.00

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Hillenbrand, G

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/15464

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 29231 A (GENZYME CORP) 2 November 1995 (1995-11-02)  the whole document ---	1-8, 10-12, 52-58
X	EP 0 213 908 A (HANA BIOLOG INC) 11 March 1987 (1987-03-11) claims 1-20; table E ---	43-51
Y	WO 97 39107 A (UNIV TORONTO) 23 October 1997 (1997-10-23) the whole document ---	43-51
Y	WO 94 08702 A (UNIV UTAH) 28 April 1994 (1994-04-28) the whole document -----	43-51



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/15464

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:  
1-12, 43-58
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/15464

### FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-12, 52-58

The subject-matter of claims 1-12 and 52-58 is directed to a cell culture medium for cells comprising an effective amount of polar amino acids selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid; a method for metabolically refeeding stored cells comprising providing a container of stored cells, adding a culture medium as defined above to the container and incubating the container of stored cells; and a method of protecting cells during isolation of the cells after enzymatic digestion of cell tissue comprising collecting digestate and adding the above defined culture medium to said digestate.

2. Claims: 13-20

The subject-matter of this group of claims is directed to a cell culture medium comprising at least one nitric oxide inhibitor.

3. Claims: 21-42, 59-60

The subject-matter of this group of claims is directed to a hydrogel matrix for cellular tissue comprising gelatin and polar amino acids selected from arginine, lysine, histidine, glutamic acid, and aspartic acid and a method of maintaining viability and functioning of cells during storage comprising embedding the cells in such a hydrogel matrix.

4. Claims: 43-46, 47-51

The subject-matter of this group of claims is directed to a transplant for implementing in a host organism comprising cells encapsulated by a matrix of totally undefined material comprising arginine, lysine, histidine, glutamic acid, and aspartic acid and a method for increasing insulin production in such a transplant encapsulated by such a matrix.

Documents W098/16629, W098/04681 and US-A-4657866, for example, disclose already a cell culture medium comprising an effective amount of polar amino acids, said polar amino acids selected from the group consisting of arginine, lysine, histidine, glutamic acid, and aspartic acid. Thus, the four different groups of inventions listed above do not belong to a common single inventive concept (no technical

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/15464

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

feature left over the known prior art - lack of unity a posteriori). Furthermore, the subject-matter of the second invention (claims 13-20 - a cell culture medium comprising at least one nitric oxide inhibitor) is not so linked together with the residual inventions as to form a single general inventive concept (lack of unity a priori).

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/15464

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9816629 A	23-04-1998	AU 4751797 A EP 0939797 A	11-05-1998 08-09-1999
WO 9804681 A	05-02-1998	AU 3740197 A CA 2261292 A EP 0920490 A	20-02-1998 05-02-1998 09-06-1999
US 4657866 A	14-04-1987	NONE	
EP 0481791 A	22-04-1992	AU 645615 B AU 8591591 A CA 2053586 A JP 2625302 B JP 6070757 A NZ 240248 A US 5633162 A US 5316938 A ZA 9108249 A	20-01-1994 07-05-1992 18-04-1992 02-07-1997 15-03-1994 25-11-1994 27-05-1997 31-05-1994 16-04-1993
WO 9529231 A	02-11-1995	AU 702402 B AU 2359895 A CA 2186608 A EP 0763102 A JP 9512171 T US 5512477 A US 5691202 A	18-02-1999 16-11-1995 02-11-1995 19-03-1997 09-12-1997 30-04-1996 25-11-1997
EP 0213908 A	11-03-1987	AU 589438 B AU 6174986 A JP 62079065 A US 4902295 A US 4997443 A	12-10-1989 05-03-1987 11-04-1987 20-02-1990 05-03-1991
WO 9739107 A	23-10-1997	AU 2283497 A CA 2179341 A EP 0894127 A	07-11-1997 13-10-1997 03-02-1999
WO 9408702 A	28-04-1994	US 5262055 A AT 162423 T AU 5166193 A CA 2147261 A DE 69316636 D DE 69316636 T EP 0664729 A ES 2111775 T GR 3026399 T JP 8504615 T	16-11-1993 15-02-1998 09-05-1994 28-04-1994 26-02-1998 07-05-1998 02-08-1995 16-03-1998 30-06-1998 21-05-1996